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The Osteogenic Potential of the Neural Crest Lineage May Contribute to Craniosynostosis

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Keywords

Craniosynostosis · Dura mater · Neural crest · Osteogenesis · Sutures

Abstract

The craniofacial skeleton is formed from the neural crest and mesodermal lineages, both of which contribute mesenchymal precursors during formation of the skull bones. The large majority of cranial sutures also includes a proportion of neural crest-derived mesenchyme. While some studies have addressed the relative healing abilities of neural crest and mesodermal bone, relatively little attention has been paid to differences in intrinsic osteogenic potential. Here, we use mouse models to compare neural crest osteoblasts (from frontal bones or dura mater) to mesodermal osteoblasts (from parietal bones). Using in vitro culture approaches, we find that neural crest-derived osteoblasts readily generate bony nodules, while mesodermal osteoblasts do so less efficiently. Furthermore, we find that co-culture of neural crest-derived osteoblasts with mesodermal osteoblasts is sufficient to nucleate ossification centres. Altogether, this suggests that the intrinsic osteogenic abilities of neural crest-derived mesenchyme may be a primary driver behind craniosynostosis.

Craniosynostosis (CS), the premature fusion of the cranial sutures, is a widely prevalent congenital malformation, affecting, on average, 1 in 2,000–2,500 live births [Boulet et al., 2008]. The head in children with CS is often deformed, as a consequence of the compensatory growth at the remaining patent sutures, in an effort to accommodate the expanding brain [Morriss-Kay and Wilkie, 2005]. In most cases, the anomalies are isolated (nonsyndromic); however, approximately 25% of the defects are associated with over 180 different sydromes [Kimonis et al., 2007; Greenwood et al., 2014]. In addition to the cranial and facial dysmorphisms that result from CS, other morbidities include increased intracranial pressure, potential developmental delay and, often, cognitive impairment [Kimonis et al., 2007]. Nonsyndromic CS is multifactorial and most commonly affects the patency of sagittal or coronal sutures [Hunter and Rudd, 1976, 1977]. Metopic, lambdoid, or multiple suture fusion accounts for less than 25% of nonsyndromic cranyosinostosis [Lajeunie et al., 1998].

Previous lineage tracing studies have described a predominance of neural crest-derived mesenchyme in the large majority of the cranial sutures [Jiang et al., 2002; Yoshida et al., 2008; Wu et al., 2017]. Importantly, the maintenance of an undifferentiated and unossified mes-

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enchyme is required for suture patency and often coincides with the establishment of a neural crest/mesoderm boundary, as clearly observed in the coronal suture, where frontal and parietal bones meet [Merrill et al., 2006]. Moreover, abnormal proliferation and premature differentiation of the sutural mesenchyme are likely the most important cellular mechanisms leading to CS [Iseki et al., 1999]. These biological events are tightly regulated by intricate signalling pathways, originating from adjacent tissues with regional and temporal control over the suture fusion sequence [Bradley et al., 1996]. For instance, the underlying dura mater has been implicated in specific determination of suture patency and ossification [Levine et al., 1998; Greenwald et al., 2000].

Rodents have been repeatedly used as a model for premature fusion of cranial sutures. Interestingly, only the posterior part of the frontal suture in mice fuses in early postnatal life, while all other cranial sutures remain patent throughout life [Bradley et al., 1996]. Further studies in rats showed that a simple change of location to where the posterior frontal suture originally lies was enough to induce the fusion of the sagittal sutures. The frontal suture, when transplanted to the anatomical position of the sagittal suture, remained patent as the latter would [Levine et al., 1998]. The expression of osteogenic markers was potently upregulated in the dura mater underlying the closing posterior frontal suture, whereas the dura mater cells under the patent sutures show significantly increased proliferation [Greenwald et al., 2000]. The dura mater underlying the coronal suture was shown to be important for maintenance of patency as removal of the membrane results in osseous obliteration in coronal suture transplants [Opperman et al., 1993]. All together, these studies suggest that not only does the dura mater directly contribute osteoprogenitors to the ossification of the frontal suture, but the meninx juxtaposed to the cranial bones also provides regionally specified signals capable of inducing fusion of normally closing sutures or maintenance of patency in certain areas. The investigation of molecular events involved in physiological suture fusion is fundamental to the understanding of pathological premature obliteration as seen in CS anomalies.

Dysfunctional suture fusion regulation performed by the dura mater has been previously proposed as an underlying cause for congenital CS. Specifically, a colony of rabbits that models CS had the synostosed suture osteotomized and a patent suture transplanted to the site of the defect. The fusion of this suture was prevented by isolating the transplant from the underlying dura mater with an impermeable material, whereas synostosis occurred when no barrier was inserted [Cooper et al., 2012]. This suggests that the CS-inducing signal was coming from the underlying dura mater and not from the cranial bones or the sutures. Other studies have implicated fibroblast growth factor as one amongst many potential dura mater-derived signals responsible for causing CS [Ang et al., 2010]. Nonetheless, the origins and mechanisms behind CS as well as the osteogenic abilities of the distinct cranial populations still remain poorly understood.

Here, we investigate the in vitro osteogenic capabilities of the distinct populations in the cranial complex, hypothesizing that the intrinsic potential of cells from different embryonic origins correlates with the potential for CS. Ectopic suture fusion in ciliopathic mice, for instance, has been previously associated with aberrant expansion of the neural crest-derived tissues at the expense of mesodermal bones [Tabler et al., 2016]. Utilizing neural crest-specific lineage tracing techniques, we compared the bone forming ability of frontal and dura mater osteoprogenitors to mesodermally derived parietal cells.

Materials and Methods

Animal Procedures

Mouse strains: *Wnt1-Cre* [Danielian et al., 1998], *Mesp1-Cre* [Saga et al., 1999], *Rosa26R*^{mTmG} [Muzumdar et al., 2007], or *Rosa26*^{Tomato} [Madisen et al., 2010] mouse lines have all been described previously. Genotyping was performed as described in original publications. All the mouse lines have been bred on a mixed background, unless otherwise noted.

Cryosectioning

Postnatal day 0 (PN0) mouse heads were harvested at birth. The samples were then fixed in 4% paraformaldehyde for 48 h at 4°C. After 3 PBS washing steps, the heads were embedded in optical cutting temperature (OCT) compound (CellPath®) in 3 steps. First, the samples were moved to a 30% sucrose solution in PBS (~24 h). Then, the embedding solution was replaced with a 30% sucrose solution mixed with OCT compound (1:1) and incubated at 4°C. Finally, the samples were moved and oriented in a plastic Tissue-Tek® Cryomold® filled with OCT compound. The mold was quickly moved into a dry ice bath with absolute ethanol until the OCT block was fully solidified. OCT-embedded samples were stored at −80°C and reconditioned to −26°C, inside the cryostat chamber, 30 min prior to sectioning. Cryosections were performed using OFT5000® cryostat microtome (BrightInstruments®). 25 µm sections were immediately mounted into Superfrost Ultra Plus® slides (ThermoScientific®), which were then stored at room temperature for future use.

Primary Cell Harvest

Dissections of postnatal calvaria were performed in PBS. Dura mater and periosteum were removed with a forceps prior to dissection of frontal and parietal. The sutures were trimmed off with

micro scissors to avoid any mixing of the bone osteoblasts with mesenchymal progenitor populations. The mineralized matrix was then digested for 10 min with 0.5% Trypsin (Sigma-Aldrich), 20 min with 2 mg/mL Dispase II (Roche) in Hank's Balanced Salt Solution (HBSS) (Gibco®) and 2× 30 min with 2 mg/mL Collagenase (Sigma-Aldrich) in HBSS. Each of the collagenase digestion steps was collected into tubes containing equivalent amount of FBS. After neutralization with FBS and mild centrifugation (1,000 rpm), the cell pellet was resuspended in 10 mL of osteoblast culture media (see formula below) and plated into 10 cm^2 dishes. Plates were incubated at 37°C and 5% CO_2.

Dura mater and interfrontal suture (IFS) cells were obtained by plating pooled dissected dura mater membranes, or IFS, after 10 min digestion with 0.5% Trypsin. The tissue was then incubated at 37°C and 5% CO_2 in osteoblast culture media.

Fluorescence-Activated Cell Sorting

Single calvariae, devoid of periosteum and dura mater, were digested according to the protocol above, and the first and second collagenase pools were collected into tubes with FBS for neutralization. After centrifugation at 1,000 rpm, the pellet was resuspended in 500 μL of PBS supplemented with 5% of FBS. The suspension obtained was pipetted through 40 μm tip strainers (Flowmi™) to obtain a single-cell suspension. The mixed membrane GFP (mGFP) and membrane Tomato (mT) cell suspension was then sorted into green and red populations using BD FACS™Aria II sorter (BD® Biosciences).

Cell Culture

Osteoblast Culture Medium

The osteoblast culture medium consisted of Minimum Essential Medium Eagle Alpha Modification (Alpha-MEM) with UltraGlutamineTMI, deoxyribonucleosides, and ribonucleosides (Lonza®); 10% batch tested FBS, and Antibiotic-Antimycotic (Gibco).

Osteogenic Medium

The osteogenic medium contained Alpha-MEM (Lonza), 10% batch tested FBS, Antibiotic-Antimycotic (Gibco), 0.25 mM ascorbic acid (Sigma), and 5 mM β-glycerophosphate (Invitrogen®).

Alkaline Phosphatase Staining

Alkaline phosphatase (ALP) activity was detected after fixation of the cultured cells with 10% paraformaldehyde. After 3 PBS washes, the culture plates were incubated for 1 h at room temperature in ALP staining solution: Sigma-Aldrich Fast Red Violet LB Salt (60 mg) (Sigma) and Naphtol AS-Mx-PO4 (10 mg) (Sigma) in 0.1 M Tris pH 8.3 (100 mL).

Von Kossa Staining

Von Kossa staining was used for detection of mineralized nodules after fixation in paraformaldehyde and ALP staining. After 3 ddH_2O washes, the culture plates were incubated in 2.5% of silver nitrate in water for 1 h under UV exposure (252 nm).

Microscopy and Image Analysis

A stereoscope (Nikon SMZ1500) with an attached camera (Nikon digital sight DS-Fi1) was used to take all the whole mount pictures and fluorescent pictures at low magnification (as indicated). Confocal microscopy was performed on a Leica Microsystems CMS GmbH TCS SP5 DM16000. Image sequences were reconstructed using FIJI (Image J) analysis software. Pixel density analysis of the staining was performed using the Analyze Particles function under the menu Analyse in the Image J software. This analysis is only possible after adjusting the threshold of the binary image created. The cell counts were performed using the plugin Cell Counter available on the Image J website.

Statistical Analysis

p values were determined using multiple unpaired *t* test analysis in the GraphPad Prism 7 Software. Every cell culture experiment was performed in triplicate (3 wells each from cells isolated from pooled animals), unless otherwise noted.

Results

Wnt1-cre Driver Identifies the Different Embryonic Origins of the Cranial Components

The embryonic origins of the mammalian skull have been previously mapped using the neural crest-specific *Wnt1-cre* driver and the *Rosa26R^{lacZ}* reporter [Jiang et al., 2002; Wu et al., 2017]. To assess in detail the neural crest and mesodermal contributions to the cranial complex, we analyzed newborn pups (PN0) using the neural crest-specific *Wnt1-cre* driver in association with a *Rosa26^{mTmG}* reporter. In these mice, cells ubiquitously express an mT marker. Upon expression of the Cre recombinase, cells switch from expression of mT to mGFP specifically in neural crest tissues. We confirmed that most of the craniofacial skeleton expressed mGFP, indicating derivation from the neural crest lineage; one exception is the parietal bones, which continue to express mT (Fig. 1A, B). As we dissected the cranial vault, we saw a predominant neural crest contribution to the sagittal suture, contrasting to a mixed contribution from neural crest and mesoderm to the coronal suture, where frontal and parietal bones meet (Fig. 1C, D). A closer look at the coronal suture reveals a clear neural crest-mesoderm boundary between frontal and parietal bones, which is underlined by a fully neural crest-derived dura mater. The overlying periosteum appears to be of mixed embryonic origin (Fig. 1E). In the original study describing compound *Wnt1-cre; Rosa26R^{lacZ}* embryos, expression of beta-galactosidase is seen in migrating neural crest cells and their mature derivatives as well as in parts of the neural tube giving rise to the midbrain and the hindbrain [Jiang et al., 2000]. Coronal sections of E17.5 heads in a later study confirmed that the frontal, but not the parietal bones were stained positive with beta-galactosidase substrate, X-gal [Jiang et al., 2002]. Moreover, the meninges, including the dura mater, were positive for X-gal around the cerebral cortex, but not the meninges overlying the midbrain and the

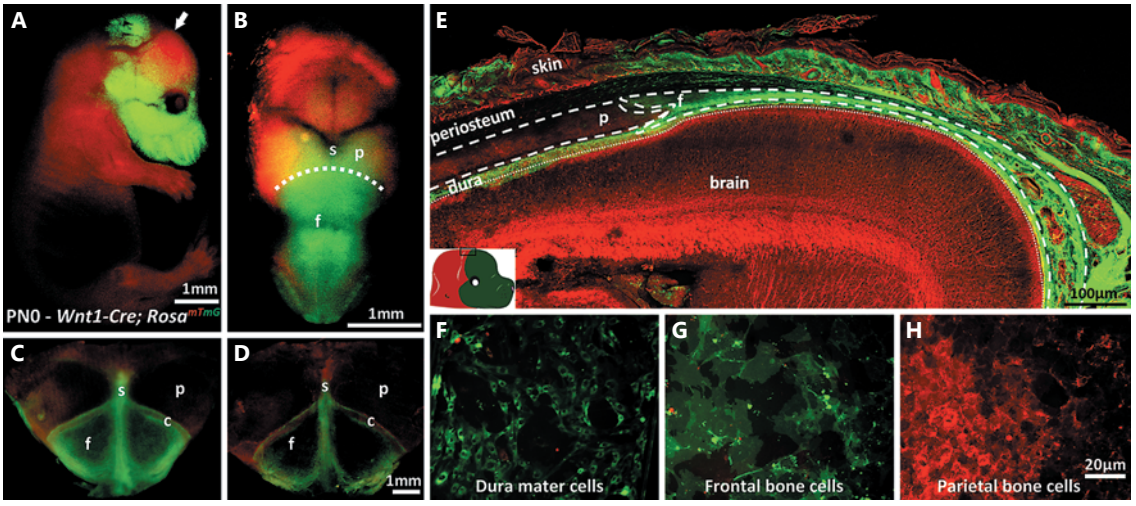

Fig. 1. *Wnt1-cre* recombination labels neural crest-derived tissues in the cranial vault. **A–D** Whole mount views of newborn (PN0) *Wnt1-cre; Rosa26R*^{mTmG} tissues. **A** Side view of the whole animal reveals predominant neural crest origin (green) of the craniofacial structure with exception of the parietal region (arrow), which is mesodermally derived. All non-neural crest tissues appear in red. **B** Top view of the head shows neural crest-mesoderm boundary (dashed line) between frontal and parietal bones. Neural crest contribution (green) is also seen at the interparietal bone and on the midline between paired parietals. **C**, **D** Dissected cranial vaults with adjacent membranes intact (**C**) or after periosteum and dura mater removal (**D**) reveal the neural crest origin (green) of frontal bone and the whole sagittal suture and mesodermal contribution (red) of parietal bones and part of the coronal suture. **E** Sagittal section at the coronal suture shows the neural crest-mesoderm boundary in detail (**Inset**). The neural crest frontal bone overlaps the mesodermally derived parietal (dashed lines) at the coronal suture, whereas dura mater is exclusively neural crest. **F**, **G** Primary cells isolated from dissected calvarial tissues and cultured for 24 hours. Dura mater (**F**) and frontal bone cells (**G**) are neural crest derived (green) and parietal cells (**H**) are mesodermal in origin (red). c, coronal suture; f, frontal; ip, interparietal; p, parietal; s, sagittal suture.

hindbrain. This is difficult to distinguish in sagittal sections in our *Wnt1-cre; Rosa26*^{mTmG} mice; however, contribution of mesodermal derivatives confirmed these observations (see below, online suppl. Fig. 1; for all suppl. material, see www.karger.com/doi/10.1159/000493106).

Because non-neural crest tissues (ectoderm, mesoderm, and endoderm) could not be distinguished using the *Wnt1-cre* genetic model, we also confirmed the mesodermal origin of the parietal bones using the mesoderm-specific *Mesp1-cre* driver. The original study describing this model, showed the neural crest origin of the meninges underlying the frontal and parietal bones [Yoshida et al., 2008]. However, this study revealed mesodermal contribution to the meninges underlying the interparietal bone. Indeed, when we looked at our *Mesp1-cre; Rosa26*^{mTmG} model, we saw mesodermal contribution (green) to the meninges overlying the posterior regions of the brain, including the cerebellum (online suppl. Fig. 1E).

One caveat with these lineage-labelling experiments is the possibility that the *cre* transgenes may be expressed in non-neural crest tissues either due to additional domains of *Wnt1-cre* expression, through insertion site effects of the transgene, through leakiness, or other effects. For example, the *Wnt1-cre* transgene is expressed at the mid-hindbrain boundary during neurulation; therefore, it would be incorrect to state definitively that all *Wnt1-cre* positive cells are neural crest. We also cannot ensure that every neural crest cell is captured by these reporters, as expression of the *Wnt1-cre* transgene only begins as the neural crest cells are being induced. Transgenic lines can also have ectopic effects, which are difficult to predict [Lewis et al., 2013]. Nevertheless, by combining microdissection with genetic labelling/ fluorescence-activated cell sorting, we are confident that our populations are as pure as technically possible.

We then confirmed the distinct origins of the cranial components by enzymatically isolating cells from the postnatal bones and dura mater (PN0). After 1 day of culture, dura mater cells are more dense and elongated, whereas frontal bone cells appear more widely spread and sparse. These cells were isolated from the explants displayed in Figure 1C, where the tissue posterior to the parietal bones is removed, which explains why our dura ma-

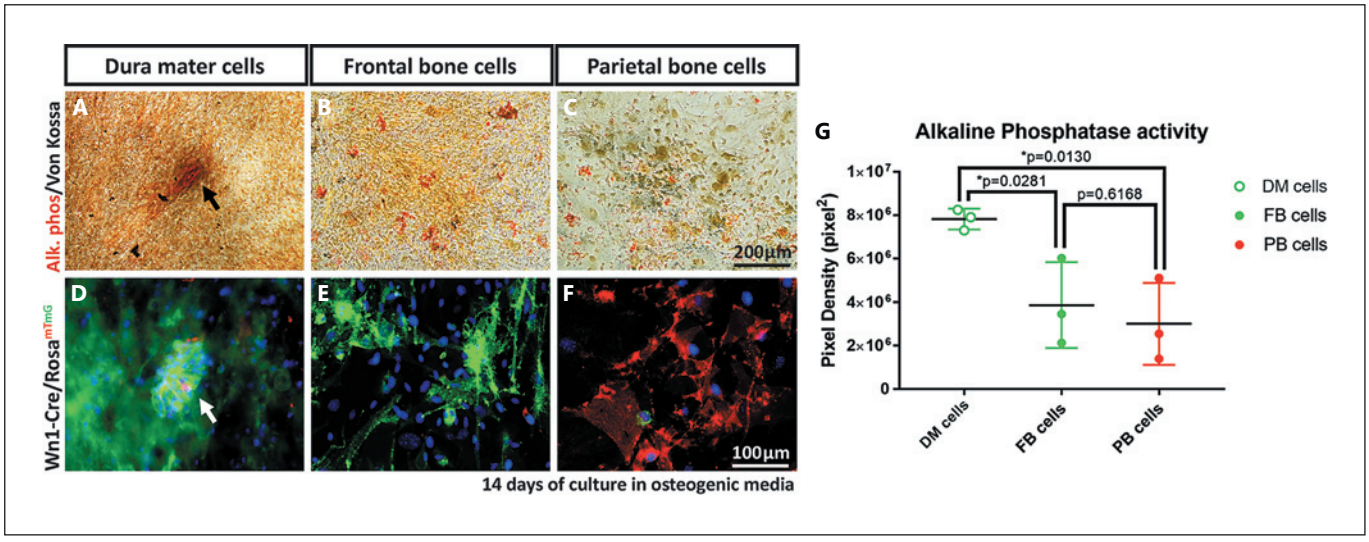

Fig. 2. Dura mater cells are more osteogenic in vitro than osteoprogenitors from the cranial vault bones. **A–C** Alkaline phosphatase (ALP) (red) and Von Kossa (black) staining of PN0 cultured cells after 14 days in osteogenic media. **A** Dura mater cells were capable of making ALP positive, mineralized, bony nodules (arrow), whereas frontal (**B**) and parietal (**C**) cells are still sparse and ungrouped at this stage. **D–F** mGFP and mT fluorescent cells in higher magnification (Hoechst – nuclei). **D** Dura mater green cells are grouped in nodule-like structure (arrow). **E** Frontal cells, although sparsely distributed, are more confluent than parietal cells (**F**) at this stage. **G** ALP activity from calvarial cells as measured by pixel density quantification of the red staining in **A–C**. Dura cells accordingly show significantly higher ALP activity when compared to frontal and parietal cells. DM, dura mater; FB, frontal bone; PB, parietal bone. * $p < 0.05$.

ter cell cultures were almost exclusively neural crest derived (Fig. 1F). Parietal bone cells resemble the morphology of frontal cells, although the well with the former was more densely populated and the cells smaller in size. (Fig. 1G, H). Although pure populations were rarely obtained when dissecting calvarial tissues, dura mater and frontal bone cells were largely green, while parietal cells were red (Fig. 1). Using this approach, these populations could be easily sorted according to their embryonic origins (online suppl. Fig. 2).

Neural Crest-Derived Dura Mater Cells Are More Osteogenic in Culture than Frontal or Parietal Osteoprogenitors

To investigate the osteogenic potential of the isolated cell populations, we cultured them at the same plating densities in osteogenic media. Fourteen days after plating, we observed the formation of mineralized nodules with intense ALP activity in the dura mater cells (Fig. 2A; online suppl. Fig. 4A). Cells derived from frontal and parietal bones, on the other hand, do not make any nodules or aggregates at this stage (Fig. 2B, C). This is accompanied by significantly lower ALP activity, as assessed by the density of the staining (Fig. 2G). The fluorescence of the cells in culture revealed that while dura mater cells are in a stage of aggregation and nodule formation (Fig. 2D), frontal cells are still sparsely distributed, and parietal cells are even more sparse and lower in density (Fig. 2E, F). This suggests that dura mater cells in culture have increased osteogenic potential in comparison to bone-derived osteoprogenitors. Moreover, a higher density and presence of aggregates in these cultures indicate an advanced stage of osteogenic differentiation, suggesting that frontal cells are at an intermediate stage between parietal cells and dura mater cells. Importantly, our criterion to determine the osteogenic potential is based on a standardized in vitro assay, which is the presence of mineralized nodules after a long period of osteogenic differentiation. While the increased mineralization could indeed be a feature of the specific cell in question, as it secretes unossified matrix and induces mineral deposition, it is also possible that the presence of more bony nodules is a consequence of increased proliferation in the earliest stages of culture, when the cells are expanding to confluency. One possibility is that cranial sub-populations isolated from the same animal reflect a difference in differentiation stage. However, regardless of the timing of isolation (e.g., embryonic stages vs. newborn or later stages, data

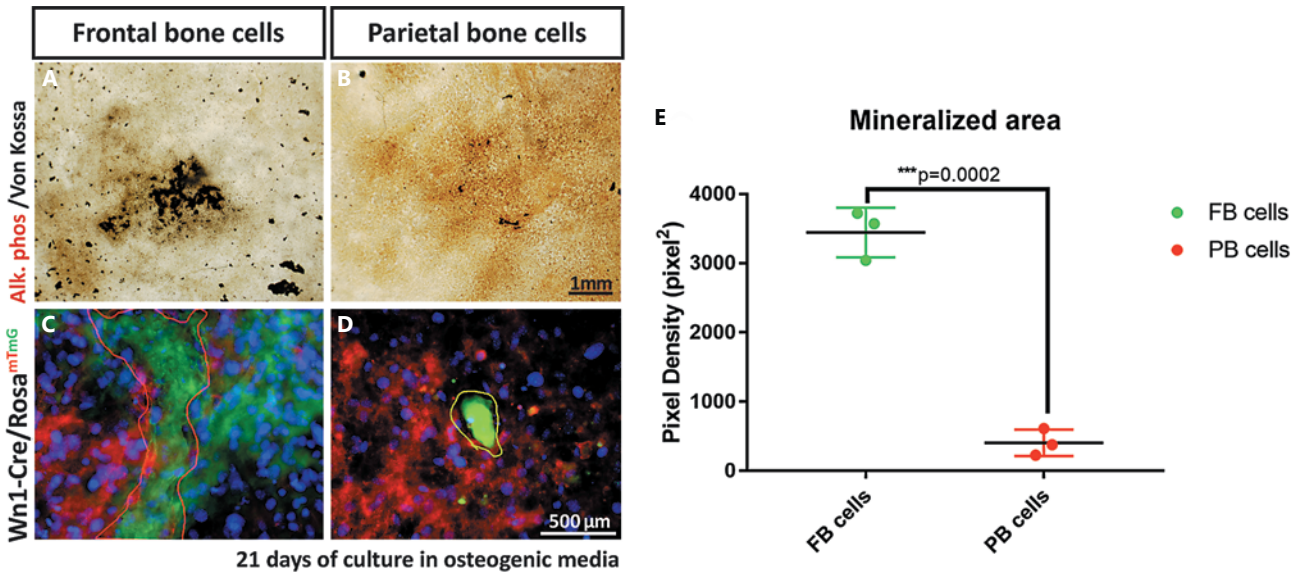

Fig. 3. Neural crest frontal bone cells are more osteogenic in vitro than mesodermally derived parietal cells. **A**, **B** Alkaline phosphatase (ALP; brown) and Von Kossa (black) staining of PN0 frontal and parietal cells after 21 days in osteogenic media. While frontal cells were largely capable of making mineralized nodules (**A**), cells isolated from the parietal bone show little mineralization and nodule formation (**B**). **C**, **D** mGFP and mT fluorescent cells in higher magnification (Hoechst – nuclei). **C** Even with the presence of non-neural crest contaminants (red cells) in the frontal cells culture, the mineralized nodules (red outline) are mostly made of neural crest-derived cells (green). **D** The rarely found nodules (yellow outline) in parietal cells culture seem to be made from neural crest contaminants. **E** Mineralized area of frontal and parietal cell cultures, as measured by pixel density quantification of black staining in **A** and **B**. Frontal bone cells yield significantly more mineralized cultures than parietal cells. FB, frontal bone; PB, parietal bone. *** $p < 0.001$.

not shown), *Wnt1-cre* positive osteoblasts appear more osteogenic, arguing against a shift in the timing of osteoblast maturation and favouring a model where the dura mater, frontal and parietal populations each contain differing proportions of osteoblast precursors.

Neural Crest-Derived Frontal Osteoprogenitors Are More Osteogenic than Mesoderm-Derived Parietals in Culture

To further confirm the increased potential of frontal bone cells over parietal cells, we cultured these populations at equivalent plating densities in osteogenic media for 21 days. This was enough to yield numerous mineralized frontal nodules, but very little mineralization of parietal cells (Fig. 3A, B). Indeed, the mineralized area was significantly higher in the former as assessed by the density of (black) Von Kossa staining (Fig. 3E). While the neural crest- and mesoderm-derived cells can be sorted en masse as previously shown, we generally opted to rely on the manual dissection of the tissues to ensure minimal disruption to the cells. For that reason, there is occasionally the presence of neural crest-derived cells in parietal osteoblast cultures and vice versa. Even a small number of these contaminants is capable of expanding to a significant proportion of the total number of cells, leading to the presence of numerous red cells mixed with green cells and vice versa (see Fig. 3C, D). Nonetheless, although the frontal bone cultures also contained cells of non-neural crest origin, nodules seen at 21 days were formed predominantly from neural crest-derived progenitors (Fig. 3C). Interestingly, in the reciprocal experiment, the few nodule-like structures found in cultures of parietal cells appeared mostly green (Fig. 3D), suggesting a requirement of neural crest-derived progenitors for formation of condensations in culture of parietal cells. Fortuitously, this raises the hypothesis that parietal cells are generally incapable or inefficient at making bony nodules in vitro. As these cells can ossify efficiently in vivo, it is likely that they are receiving pro-osteogenic signals from the adjacent neural crest.

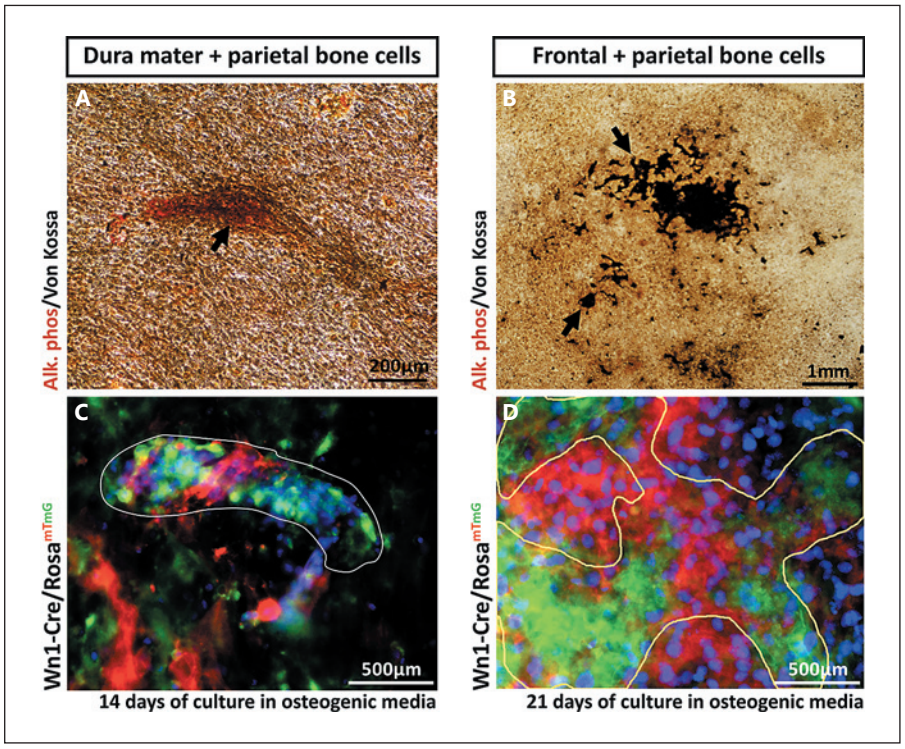

Fig. 4. Parietal bone cells can contribute to nodule formation as long as they are co-cultured with neural crest-derived cells. **A** ALP/Von Kossa staining of equivalently mixed dura mater and parietal cells reveal presence of mineralized nodules (arrow) after 14 days of culture in osteogenic media. **B** ALP/Von Kossa staining of frontal and parietal mixed cultures; after 21 days in osteogenic media they are largely mineralized (black staining, arrow). **C** mGFP/mT fluorescence reveals mixed neural crest (green) and mesoderm (red) contribution for nodule (outline) formation (Hoechst – nuclei). **D** mTmG fluorescence reveals neural crest (green) and mesoderm (red) mixed contribution for nodule formation (yellow outline).

Co-Culture with Neural Crest-Derived Cells Favours in vitro Osteogenesis of Parietal Osteoblasts

We next set out to test whether co-culturing of neural crest-derived cells and parietal progenitors at equivalent plating densities would produce bony nodules with mixed contributions. We observed that, after 14 days of culture, dura mater/parietal co-culture yielded intense ALP activity and a large number of nodules (Fig. 4A; online suppl. Fig. 5A). These nodules were mostly of mixed neural crest/mesoderm origin with no particular bias, although fluorescent labelling revealed that they appear to be nucleated by the green neural crest cells, which are slightly more abundant in dura mater/parietal cell cultures (Fig. 4C; online suppl. Fig. 3 for quantification). Moreover, the culture of frontal bone cells with parietal cells resulted in vast mineralization and abundance of bony nodules after 21 days (Fig. 4B). Accordingly, these nodules were not skewed towards neural crest nor mesodermal cells (online suppl. Fig. 4), although the base of the nodules was mostly formed by green cells (Fig. 4D). This was in contrast to the absence of mesodermally derived nodules in culture of isolated parietal cells (compare Fig. 4D and Fig. 3D). A limitation of our analysis was that the ALP and Von Kossa staining masked the fluorescence of the cells, such that the imaging of nodules and their cell content had to be performed prior to the staining procedure. Nonetheless, it would be interesting to quantify the proportion of neural crest and mesoderm cells that are also positive for ALP. Immunostaining assays will be done in the future to address this further.

All together, our study indicates that although mesodermally derived parietal osteoprogenitors seem to lack the ability to efficiently make nodules in culture, they are able to contribute to the formation of these structures when co-cultured with cells of neural crest origin, which seem to nucleate the bony nodules. This raises the need to further investigate potential signals between calvarial cells of distinct embryonic origins to improve our understanding of the molecular signalling at the neural crest-mesoderm boundary in the skull.

Dura Mater Cells Are the Most Osteogenic amongst Neural Crest-Derived Cranial Progenitors

In light of the higher propensity for osteogenesis displayed by the neural crest tissues in the cranial vault, we decided to investigate which of the site-specific progenitors would show higher osteogenic activity in vitro. PN10 osteoblasts were harvested from 3 distinct locations in *Wnt1-Cre; Rosa26*^{Tomato} mice (see schematics on Fig. 5K) and cultured for 21 days in osteogenic conditions. At day

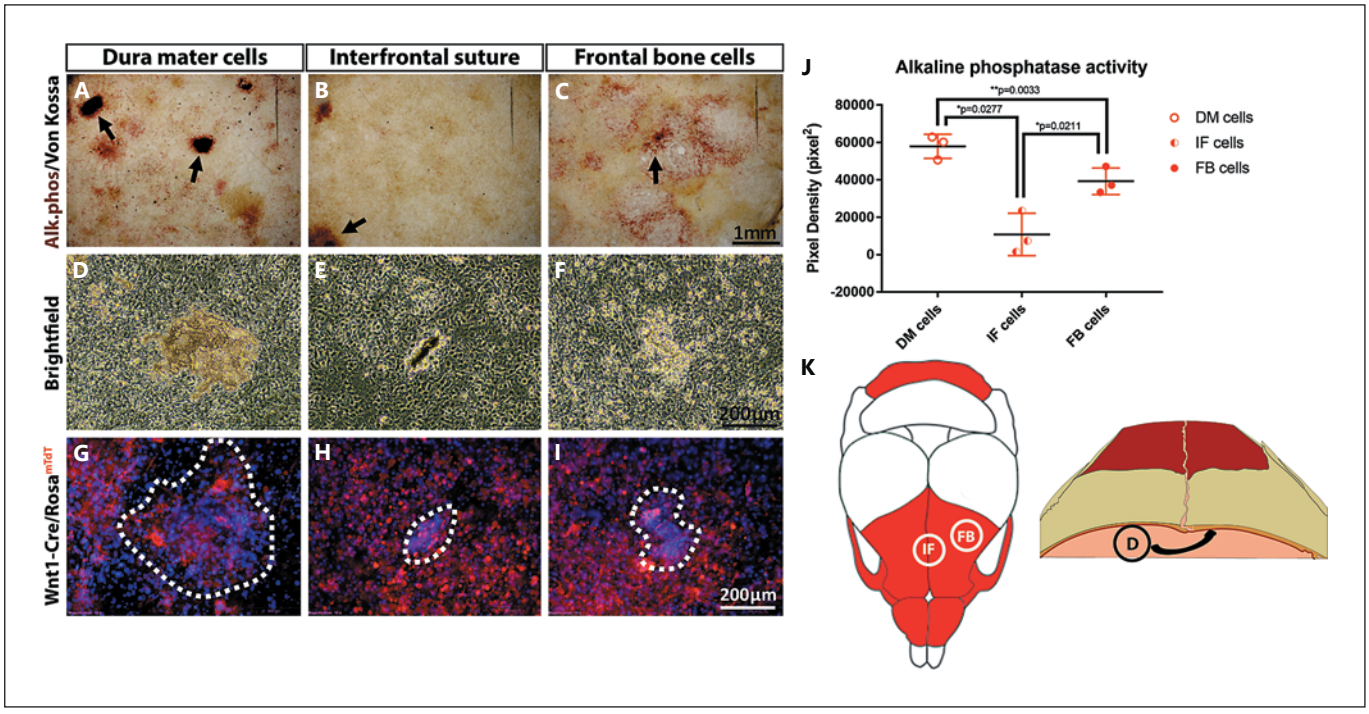

Fig. 5. Dura mater cells are the most osteogenic amongst neural crest-derived cranial populations. **A–C** ALP/Von Kossa staining of primary cells from dura mater, interfrontal suture and frontal bone, respectively. Arrows indicate mineralized nodules. **D–F** Contrast phase high magnification nodules reveal 3D-mineralized structure surrounded by a monolayer of cells. **G–I** Tomato fluorescence reveals neural crest origin of the Dura, interfrontal suture (IFS), and frontal bone (FB) populations. The nodules outlined are increased in size in dura cell cultures. **J** Pixel density quantification of ALP staining shows significantly increased activity of dura cells when compared to IFS and FB cells, the latter is also significantly higher than in IFS cells. **K** Schematics show sites of harvesting where the cells originated from. DM, dura mater; IF, interfrontal; FB, frontal bone. * $p < 0.05$; ** $p < 0.005$.

21, we find that dura mater cells are denser and possess significantly higher ALP activity when compared to cells from IFS and frontal bone osteoblasts (Fig. 5J). These cells also produce more mineralized nodules than the latter (online suppl. Fig. 5B) and in larger size (Fig. 5G–I). The presence of mineralized nodules is comparable between IFS cells and frontal bone osteoblasts (online suppl. Fig. 5B). However, the higher area of ALP staining indicates that frontal bone cultures have more progenitors committed to the osteogenic pathway, which are actively making bone in vitro (Fig. 5A–C). This might reflect an expected delay in the differentiation stage, given that a proportion of the sutural mesenchyme is still uncommitted when harvested from the explants, whereas the cells harvested from the bone chips are likely fully differentiated at this postnatal stage. While it is true that the sutural mesenchyme harbours numerous undifferentiated stem cells with multi-lineage potential [for review, see Doro et al., 2017], it is possible that, in vitro, these cells require exogenous signalling input to undergo osteogenesis. In addition, it would be interesting to know the dura mater requirements for ossification of the sutural cells.

Discussion

The embryonic origins of the cranial components are often neglected in studies using primary cell cultures for osteogenic assays. While heterogenous populations isolated from whole calvaria have been largely used for in vitro differentiation assays, the bone forming ability of calvarial osteoprogenitors appears to be site and lineage specific. Each tissue in the cranial vault harbours distinct osteogenic capacity, being implicated in different roles during development and likely having distinct contributions to bone diseases and malformations. One classic example is the investigation of the pathogenesis of CS.

Although the premature ossification of a sutural mesenchyme may ultimately influence multiple tissues of dis-

tinct embryonic origins, it is still unclear which specific cell populations are the primary drivers of pathogenesis. Rather, as studies have shown, the dura mater seems to be the major player behind the suture patency, or fusion, during skull development. Either by providing molecular signals, or by directly contributing osteoprogenitors that undergo differentiation, the underlying dura mater may determine whether a cranial suture fuses or remains patent [Mehrara et al., 1999; Greenwald et al., 2000; Warren et al., 2003]. This membrane is regionally specified and can sometimes undergo aberrant differentiation, causing severe consequences for the adjacent tissue, as in CS [Ang et al., 2010; Cooper et al., 2012], or even undergo tumorigenesis as seen in meningeal osteosarcomas [Bonilla et al., 1994; Ringenberg et al., 2000]. The understanding of the osteogenic potential and its correlation with the distinct embryonic origins is therefore fundamental for the comprehension of CS and may reveal a propensity of neural crest-derived tissues to undergo hyperossification.

As we have reproduced in this study using genetic labelling and lineage tracing, the dura mater is exclusively neural crest derived, as are the frontal bones. Even though the parietal bones are derived from the mesoderm, all the cranial sutures including the coronal are thought to be predominantly populated by neural crest osteoprogenitors. We have shown that the dura mater-derived cells are much more osteogenic in vitro than the osteoblasts harvested from frontal or parietal bones. This was revealed by an increased ALP activity and the prompt generation of bony nodules in dura mater cell cultures. Moreover, the osteoblasts derived from neural crest frontal bones showed significantly increased in vitro osteogenesis when compared to mesoderm-derived parietals. The latter were highly inefficient at making mineralized nodules in culture. However, the co-culture of parietal osteoblasts with neural crest-derived cells overcame this inefficiency, as the neural crest nucleated the bony nodules, allowing the mesodermal cells to engage in osteogenesis. This finding corroborates previous studies that report an ability of dura mater cells to provide a pro-osteogenic signal to cells in co-culture [Cooper et al., 2012].

The neural crest-derived cranial populations also showed distinct cell-autonomous osteogenic potential in vitro. We compared the ALP activity and the presence of mineralized nodules in cultures of frontal bone osteoblasts, dura mater, and IFS progenitors. The dura mater, as expected, showed the highest osteogenic capacity, possibly accompanied by an increased proliferation, judging by the higher density of cells when compared to every other cranial population. Moreover, the cells derived from frontal bone osteoblasts displayed increased ALP activity in comparison to cells from IFS. This could be indicative of a differentiation delay in the cells of the suture, which is unsurprising if we assume that the frontal osteoblasts are fully differentiated when harvested from the bone chips, while the sutural mesenchyme is still uncommited to the osteogenic lineage. Could the cells from the suture then be inherently programmed to remain undifferentiated in the absence of a pro-osteogenic cue? Do these cells require the input from the dura mater to undergo osteogenesis? Are any other lineages of a common progenitor obtained in these cultures at the expense of osteogenic cells? Future studies should include molecular analysis in order to confirm the differentiation status of the neural crest-derived cells.

The investigation of signalling cues is very important for understanding the pathophysiology of CS. A recent study, in fact, has identified a number of de novo mutations in negative regulators of the Wnt, BMP, and Ras/ERK pathways, occurring in non-syndromic midline CS patients [Timberlake et al., 2017]. These pathways, knowingly implicated in positive regulation of osteogenesis, are, among other signals previously studied [Ang et al., 2010], good candidates for dura mater's regulatory milieu in the context of suture patency and fusion. All together, our study identifies distinct populations of osteoprogenitors in the cranial vault and provides a deeper understanding on the ossifying capabilities of these cells. Our hope is that this study will aid the comprehension of general suture biology and pathogenesis.

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Statement of Ethics

All animal work was performed at King's College London in accordance with the UK Home Office Project License P8D5E2773 (K.J.L.).

Disclosure Statement

The authors have no conflicts of interest to disclose.

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